# Effects of aluminum on the release and-or immobilization of soluble phosphate in corn root tissue

**Abstract.** The effects of aluminum ions on the generation of mobile inorganic phosphate (Pi) within the cells of excised maize (*Zea mays* L.) root tips were examined using $^{31}$P-nuclear magnetic resonance ($^{31}$P-NMR) spectroscopy. When perfused with a solution containing 50 mM glucose and 0.1–5.0 mM $Ca^{2+}$ at pH 4.0, 3–5-mm-long excised maize root tips from 3-d-old seedlings showed a significant (approx. 100%) increase in the amount of mobile Pi, (primarily vacuolar) over a period of 30 h. This increase was above that which can be accounted for by the hydrolysis of endogenous sugar phosphates and nucleotides. A change of the pH of the perfusion solution to 7.0 reduced the increase in Pi to approx. 50%. Omission of $Ca^{2+}$ in the solution at pH 4.0 caused the mobile Pi to increase to about 170%. However, the presence of $Al^{3+}$ or both $Ca^{2+}$ and $Al^{3+}$ in the solution resulted in a significant loss (35–50%) of mostly vacuolar Pi over the same period of time. When root tips containing up to 65% of newly released Pi, produced after 20 h perfusion, were exposed to $Al^{3+}$, no additional increase in the level of the mobile-Pi signal area was noted. Exposure to $Al^{3+}$ with $Ca^{2+}$ and glucose under hypoxia at pH 4.0 resulted in a threefold decrease in intracellular Pi content after the root tips were returned to aerobic conditions. These results indicate that external pH plays an important role in the generation of mobile intracellular Pi and that the presence of both $Ca^{2+}$ and $Al^{3+}$ can independently suppress the production of this excess Pi and ultimately reduce the vacuolar Pi.

**Key words:** Aluminum precipitation, toxicity – Hypoxia – Phosphatase – Phosphate depletion – Phosphate, NMR-invisible – Vacuole – *Zea* (aluminum and phosphate).

*Abbreviations and symbols:* NMR = nuclear magnetic resonance; Pi = inorganic phosphate; UDPG = uridine diphosphoglucose; $\delta$ = chemical shift

## Introduction

Although it is well documented that aluminum is a ubiquitous element in soil that can be highly toxic to plants, the mechanism of this toxicity is not well understood. In-vitro studies have focused on the effects of aluminum on the ATPase of the root plasma membrane (Caldwell and Haug 1982; Tu et al. 1986), phosphorylating enzymes such as hexokinase (Trapp 1980), and the regulatory protein calmodulin (Siegel and Haug 1983; Haug 1984). However, the validity of these studies in intact tissue is always in question since isolated cell organelles, membranes and enzymes may have different sensitivities to toxic metal ions. Furthermore, the intact root cells are enclosed by a cell wall within the tissue. Thus, certain in-vivo processes which will normally be affected by cell-wall biochemistry, such as metal-ion trapping (Horst et al. 1983) and hydrolysis of phosphorylated compounds (Crasnier et al. 1985) cannot be accounted for in in-vitro experiments.

Many studies of intact plants indicate that aluminum toxicity is manifested as a general phosphate deficiency of the apical meristematic cells of the root (Foy 1984). Helyar (1978) has demonstrated that aluminum interferes with phosphorus metabolism and cell-wall elongation upon binding to lamellar pectins. These findings agree with the earlier findings of McCormick and Bordon (1972) who observed aluminum precipitation of exogenous phosphate within the cell walls and the intercellular free space of the root tissue. Earlier electron-microscopy studies of maize root cells using

energy-dispersive X-ray techniques demonstrated that aluminum was precipitated on the surface of the epidermal cells of the root (Rasmussen 1968). However, more recent work utilizing molybdenum-blue-stained whole and sectioned barley roots showed phosphate "fixation" associated with aluminum in the root-cap and cortical regions extending as far up as 5 mm from the root tip (McCormick and Bordon 1972). Santana and Braga (1977) have observed a decrease in phosphorus content in rice shoots with increasing aluminum levels in the soil, and Cambrai and Calbo (1980) have reported that pretreatment of the rods of an aluminum-sensitive sorghum cultivar with aluminum decreased phosphate uptake by 42%. Similarly, Fox (1979) noted that certain aluminum-tolerant maize cultivars were more tolerant to low phosphate soils than aluminum-susceptible cultivars. Other workers have observed cross-interactions of aluminum with calcium. Simpson et al. (1977) attributed poor root growth of alfalfa to calcium deficiency produced by aluminum. This was also suggested by Awad et al. (1976) in studies with Kikuyu grass. These authors concluded that the reduction in yield caused by aluminum was related to a specific synergism with calcium, which ultimately produced a phosphate deficiency in the plant. Lance and Pearson (1969) showed that reduced calcium uptake was the first observable symptom of aluminum damage in cotton seedling roots, and suggested that aluminum may interfere with the function of calcium in the plasmalemma. To a certain extent the results of Rhue (1979) agree with these findings since they show that the damaging effects of aluminum on Al-sensitive maize cultivars could be prevented by supplying the plants with 16-fold higher concentrations of calcium.

Recently we have demonstrated, using in-vivo phosphorus-31 nuclear magnetic resonance ($^{31}$P-NMR), that at a lowered level of metabolism, i.e. in the absence of an exogenous source of carbohydrate, excised maize root tips showed a decrease of $O_2$ consumption and severe reduction in ATP and sugar-phsophate levels when exposed to $Al^{3+}$ at low pH (Pfeffer et al. 1986). We also observed that the $^{31}$P-NMR representing the intracellular level of mobile (NMR-visible) Pi, i.e., the signal attributable to freely soluble Pi (Roberts 1984; Loughman and Ratcliffe 1984; Pfeffer 1987), as opposed to phosphate found in membrane phospholipids, nucleic acids or cell-wall-bound constituents, remained approximately constant when calcium was present. The latter forms of phosphate, because of their long rotational correlation times, are not detectable with the normal high-resolution, solution NMR methodology used for in-vivo experiments (Pfeffer 1987). Thus they must be considered as NMR-invisible pools (Brodelius and Vogel 1985; Karczmar 1985). However, in the absence of aluminum at low pH a significant increase in the mobile Pi level was observed. These results indicate that without sufficient metabolic activity in the cells, aluminum toxicity is primarily manifested by a drop in nucleotide levels, in addition to some yet to be defined interaction with incipiently generated soluble Pi.

In the present study we examine the long-term effects of aluminum on the energy status and mobile-Pi level in the cells when the tissue is continually perfused with a sufficient supply of carbohydrate and calcium ion to keep it at a high state of metabolic activity. In this context, we will discuss 1) the nature of the endogenously liberated mobile Pi in non-aluminum-stressed tissue, and 2) the apparent interference of aluminum and calcium with the release and-or cell-wall precipitation and immobilization of Pi under various conditions.

## Material and methods

*Plant material.* Maize kernels (*Zea mays* L. cv. FRB-73; Illinois Foundation Seeds, Champaign, Ill., USA [1]) were germinated in a growth chamber at 28° C for 72 h as previously described in Pfeffer et al. (1986). For each experiment, approx. 700–900, 3–5-mm-long root tips were required; these were generally examined by $^{31}$P-NMR in the perfused state within 1 h of excision.

*Experimental solutions.* Solutions were prepared from highly purified deionized water. The perfusion system was thoroughly cleaned with approx. 1% NaOCl solutions (diluted commercial bleach) and rinsed clean before each experiment. All solutions at pH 4 were unbuffered and contained from 0.1 to 5 mM calcium sulfate except when specified, and 50 mM glucose or 25 mM sucrose with the addition of from 0.25 to 2.5 mM aluminum sulfate. The pHs of these perfusate solutions were monitored and adjusted throughout the experiments. Solutions at pH 7.0 were buffered with 10 mM Mes (2-(N-morpholino)ethanesulphonic acid). All solutions were continually saturated with either $O_2$ or $N_2$ gas and circulted through the perfusion system with the root tips at a rate $>45$ ml·min$^{-1}$. Prior to the connection between the perfusion tubes and the NMR tube, a 30-s evacuation of the tube with a low vacuum was carried out to remove trapped gas bubbles from between the roots. This procedure was important to prevent heterogeneous broadening of the spectra. A change from one perfusate solution to another was accomplished by means of a two-way double stopcock assembly that connected two separate reservoirs to the peristaltic pump and NMR tube assembly. Prior to changing from one solution to another a 100-ml flush of the new perfusate solution was passed through the system to minimize contamination.

[1] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over other products of a similar nature not mentioned

*Nuclear-magnetic-resonance experiments.* A narrow-bore (54 mm) Japan Electronic and Optical Laboratories (Tokyo) GX-400 NMR spectrometer operating at 21–22° C was used to obtain the 161.7 MHz $^{31}P$ spectra of 700–900 excised (3–5-mm) root tips as described in Pfeffer et al. (1986). In order to carry out perfusion-NMR experiments safely (without leakage hazards) for extended periods of time (up to 48 h) inside the magnet, an additional external suction tube was set into the reservoir of the NMR-tube spinner housing to prevent overflow of liquid (in the event of a leak at the NMR cap) onto the probe and shim coil insert. In all other respects, the perfusion system design and operation was as previously described (Pfeffer et al. 1986). A reference capillary containing 120 mM hexamethylphosphoramide (HMPA) was used to give a satisfactory size reference peak for each spectrum. The HMPA exhibited a resonance at 30.73 $\delta$ downfield from 85% $H_3PO_4$. All chemical shifts were referenced relative to 85% $H_3PO_4$ which was assigned a value of 0.0 $\delta$.

The fast acquisition accumulation parameters, 30° pulse (12 μs), 2000 data points zero filled to 16000, recycling time of 0.162 s, 10000–40000 transients/spectrum, 16 kHz spectral widths and 15 Hz line broadening were used for all experiments to examine relative changes in Pi and organo-phosphorus components. Slow recycling-time parameters, 90° pulse and 20 s recycling time were used to establish the quantitative relationships, i.e. the response factors for each resonance (Schleich et al. 1984) among the components within each spectrum. From this information we were able to correct for the distortion of the areas seen in the rapidly acquired spectra. In the rapidly acquired spectra, the resonances associated with long relaxation times (2.8–3.6 s), i.e. sugar phosphates and Pi in the cytoplasm and vacuole, were suppressed by 52% relative to the rapidly relaxing resonances (0.3–0.6 s) associated with nucleoside triphosphate (NTP) and uridine diphosphoglucose (UDPG).

In order to evaluate the levels of mobile Pi in the tissue over the period of these sequential experiments, we utilized the fast acquisition response factors for each resonance, i.e. sugar phosphates, Pi (vacuolar and cytoplasmic), NTP and UDPG. At each time interval, the level of Pi was determined on the basis of the contribution to Pi produced from the decrease (assuming complete hydrolysis) of sugar phosphates, NTP and UDPG. It is assumed that negligible amounts of nucleotide diphosphate (NDP) and nucleotide monophosphate (NMP) are present at any time. Conversion of one molecule of NTP to Pi results in a Pi resonance response equal to three times the observed loss of the $\gamma$-NTP resonance area ($-4.60\ \delta$) divided by the response factor 2.08. This factor is derived from the relaxation difference between the $\gamma$-NTP and vacuolar-Pi resonance (Pfeffer et al. 1986). Similarly, the contributions to Pi coming from UDPG hydrolysis are calculated using the change in the area of $-11.74\ \delta$ resonance times 2 divided by 2.08. Response factors for the sugar-phosphate resonances were essentially equal to those for the Pi resonance. Thus, losses in these areas were equated directly to increases in Pi areas with no adjustments in intensity. When line widths were uniform throughout a sequence of spectra (16–17 over a period of 34 h), we found little measurable difference in the relative changes in compound levels from either area or line-intensity measurements. The following equation gives the method described for calculating the change in mobile-Pi concentration over and above that produced from nucleotide and sugar-phosphate hydrolysis:

$$\%\ \text{change} = 100 \cdot ([\text{vac Pi}_t + \text{cyt Pi}_t] - 3[\gamma\text{NTP}_t - \gamma\text{NTP}_o]/2.08 - 2[\text{UDPG}_t - \text{UDPG}_o]/2.08 - [\text{sugar-Pi}_t - \text{sugar-Pi}_o])/[\text{vacPi}_o + \text{cyt Pi}_o]$$

where o = concentration at the initial time, t = concentration at time t, cyt Pi = cytoplasmic orthophosphate, vac Pi = vacuolar orthophosphate.

The average change in mobile Pi concentration for successive experiments was plotted against time for each change in conditions indicated. The average standard deviation for three replicate experiments was no greater than 14%.

The amounts of mobile phosphorus compounds in the root tissue samples, averaged over the total sample volume within the detector coils, were determined by comparing the area of the signal from the HMPA resonance (observed in the tissue spectra) to the area response given by the phosphorus resonances for 1 mM standard solutions of ATP, glucose 6-phosphate and Pi in the same sample volume. Both the tissue and the standard solution spectra were acquired under quantitative conditions. Adjustments were then made for differences in signal responses (see above) to establish a direct relationship between the area of the resonances obtained under the fast and slow acquisition regions. A typical spectrum obtained from approx. 800 tips exhibited a concentration profile (within the coil volume of the probe of 1.6 ml) of approx. 1.0–2.0 mM sugar phosphates, 0.6–0.7 mM cytoplasmic Pi, 1.0–3.0 mM vacuolar Pi, 0.3–0.5 mM NTP, and 0.3–0.5 mM UDPG + NAD depending upon the age and size of the tips used. Spin lattice relaxation times were determined by the inversion recovery method (180-$\tau$-90°) with 16-s repetition times for the non-nucleotide resonances and 4 s for the nucleotide resonances between scans, respectively. Relaxation values were calculated using a two-parameter exponential fit. The $T_1$ values, as reported earlier (Pfeffer et al. 1986), for both nucleotide and non-nucleotide resonances at the initiation and completion of a 34-h sequence of experiments were found to be identical within experimental error.

*Estimation of pH.* Estimates of intracellular pH from cytoplasmic and vacuolar phosphate were made using the standard reference curve of pH versus $^{31}P$ chemical shift in PPM (Roberts et al. 1982; Pfeffer et al. 1986). Solutions prepared for the evaluation of this pH profile contained 5.0 mM $K_2HPO_4$, 2.0 mM $MgCl_2$ and 100 mM KCl. As above, all shifts were referred to HMPA in a reference capillary with a chemical shift position of 30.73 $\delta$. From these profiles, intracellular pH could be estimated with an accuracy of $\pm 0.1$ pH unit (Roberts et al. 1981). The resonance assignments for cytoplasmic and vacuolar Pi have been made by examining different lengths of excised maize root tips by $^{31}P$-NMR (Roberts et al. 1980). Since the cells of 1–2-mm root tips contained small vacuoles and those of 5–10-mm tips large vacuoles, the derived spectra from each of the populations gave a phosphate resonance intensity corresponding to its dominant compartment (cytoplasmic or vacuolar, respectively). Verification that the cytoplasmic-phosphate resonance corresponds to the pH 7.7 was made by comparing its and the glucose 6-phosphate resonance pH shift profile. Only very small changes, <0.1 pH unit, were observed in the cytoplasmic pH over the duration of experiments up to 34 h. Because of the insensitivity of the chemical shift of the vacuole Pi at pH approx. 5.5 (Roberts et al. 1982), no measurable change in the vacuolar pH could be detected throughout these experiments.

*Measurement of acid-phosphatase (EC 3.1.3.2) activity.* About 20–30 maize root tips were incubated in 1.9 ml of a solution containing 50 mM Na-acetate, pH 4.2, for 10 min at 22° C. The reaction was started by the addition of 100 μl of 160 mM Na–*p*-nitrophenol phosphate to the root-tip suspension. After 20–40 min, the reaction was quenched by the addition of 2 ml of 1.0 M $Na_2CO_3$ (final pH 10–10.2). The acid-phosphatase ac-

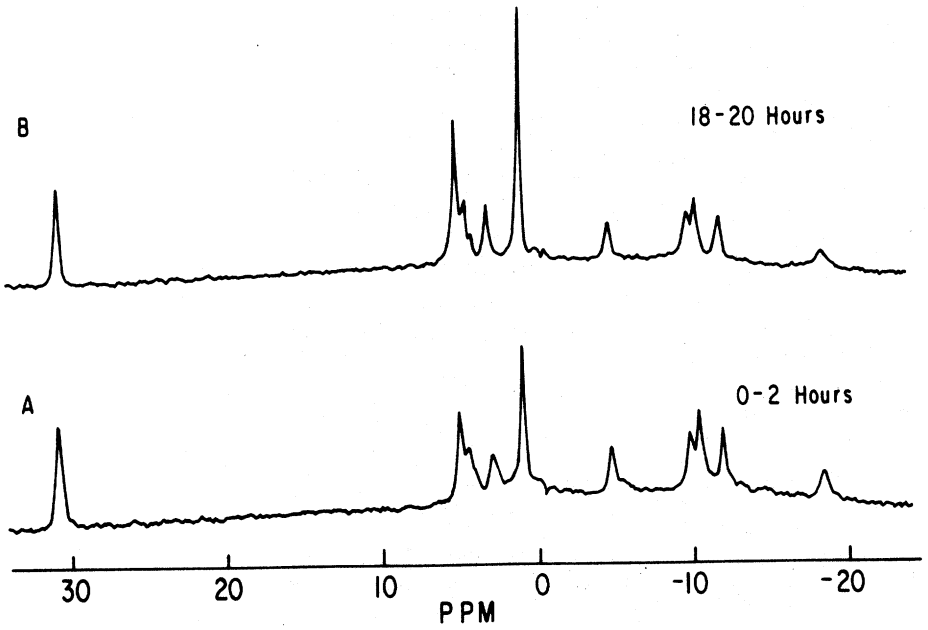


**Fig. 1 A, B.** 161.7 MHz $^{31}P$ spectra of 700–900 3–5-mm root tips from 3-d-old maize seedlings perfused with a solution containing 50 mM glucose, 0.1 mM $CaSO_4$ at pH 4.0 for 0–2 h (**A**) and 18–20 h (**B**). Each spectrum is the result of 40000 scans (2 h). The reference is relative to 85% $H_3PO_4$ and the low field resonance is from 120 mM hexamethylphosphoramide in a capillary. The assignments are as follows: sugar phosphates 5.08–3.91 $\delta$; cytoplasmic Pi 3.0 $\delta$; vacuolar Pi 0.89 $\delta$; $\gamma$NTP–4.80 $\delta$; $\alpha$NTP $-9.50\ \delta$; UDPG $-10.29\ \delta$; UDPG $-11.74\ \delta$; $\beta$–NTP, $-18.26\ \delta$

tivity was determined from the absorbance of a quenched solution at 405 nm using an molar absorptivity for *p*-nitrophenolate of 8.71 $mM \cdot cm^{-1}$. The root tips were then collected by filtration and dried overnight in an vacuum oven (25° C) for weight determination.

*Aseptic experiments and detection of microbial contamination.* Aseptic NMR experiments were performed on surface sterilized (5 min submersion in ethanol and wash with distilled water) kernels that were grown for 3 d in autoclaved glassware and paper. All glassware and metal instruments used for perfusion were also autoclaved. The perfusion system, i.e. all tubing and fittings, were cleaned with 11% NaOCl solutions, rinsed with a dilute solution of HCl and finally with distilled water.

Microbial samples of the perfusate were taken at zero time and 24 h and 36 h after the experiments were begun. Malt-extract agar (MEA) and solutions LB (Luria-Bertani) and M9 were prepared according to the protocol of Maniatis et al. (1982). Colony counts were recorded at 24 h and 72 h.

For plating, samples were brought to sixfold dilutions $10^{-1}$–$10^{-5}$ and 0.1 ml of each diluted original sample was spread-plated with a plate dilution of $10^{-1}$–$10^{-6}$. The colony count was then recorded and multiplied by the magnitude of the dilution to find the number of microbes in the original 1-ml sample. Dilution plates with colony counts $>20$ and $<200$ were most accurate for the calculation of the number of organisms present in the original solution.

## Results

*General considerations of the $^{31}P$ spectra of maize root tips.* Figure 1 A and B are representative 161.7 MHz $^{31}P$ spectra of 700–900 maize root tips after 2 and 20 h of perfusion with 0.1 mM $CaSO_4$ and 50 mM glucose at pH 4.0. The assignments for these spectra are as previously reported (Pfeffer et al. 1986) and are given in the figure legend. The ratio of cytoplasmic/vacuolar Pi in the initial spectra varies as a function of the morphology, age and size of the root tips chosen (Roberts et al. 1980). The ratio of nucleotide to non-nucleotide resonances represented by these spectra are distorted because of the rapid recycling time used in these experiments (see *Material and methods*). In fact, the nucleotide level represents less than 15 mol% of the observable mobile phosphorus compounds examined. This means that even major changes in the nucleotide-species resonance area (such as complete hydrolysis to Pi) have a relatively minor effect on the Pi resonance area. The pH of the cytoplasm (Pi shift) corresponding to a chemical shift of 3.0 $\delta$ is $7.50 \pm 0.10$ using a perfusate at pH 4.0. When the external pH was raised above 6.0, the cytoplasmic pH generally moved to pH 7.6–7.8. Over this external range of pH (4.0–6.0) there was no observable change in the vacuolar pH. In general, over long periods of time up to 34 h at pH 4.0, there appeared to be little change in cytoplasmic pH ($<0.1$ pH unit). Except for conditions of hypoxia, the level of cytoplasmic Pi remained nearly constant, while the vacuolar Pi level varied depending on the externally controlled environment. After approx. 1 h of perfusion with 50 mM glucose solution, the sugar-phosphate resonance intensity increased by 20% and the ratio of sugar phosphates increased in favor of glucose 6-phosphate at 5.08 $\delta$.

Over a period of 20 h, nucleotide levels dropped by 18–20% with the standard conditions of 50 mM glucose, 0.1 mM $CaSO_4$ and pH 4.0

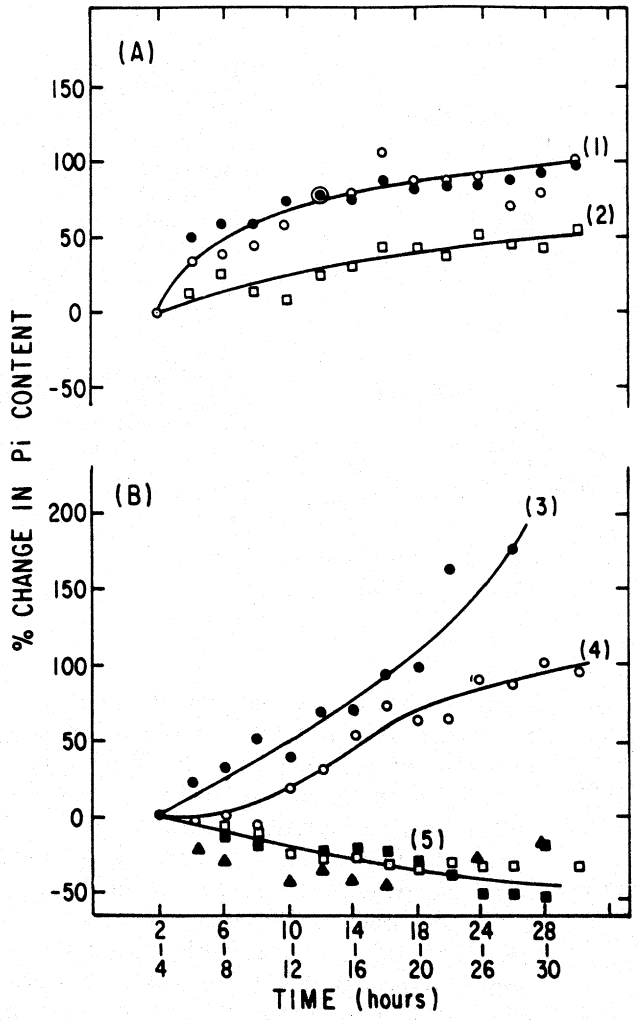


**Fig. 2A, B.** Change in content of NMR-visible Pi signal from $^{31}P$ spectra of 3-d-old corn root tips with time. **A** pH effects: *trace 1* ○ 0.50 mM glucose + 0.1 mM $Ca^{2+}$, pH 4.0, and ● 25 mM sucrose + 0.1 mM $Ca^{2+}$, pH 4.0; *trace 2* (□) 50 mM glucose + 0.1 mM $Ca^{2+}$, pH 7.0. **B** Metal-ion effects: *trace 3* (●) 50 mM glucose, pH 4.0; *trace 4* (○) 50 mM glucose + 5 mM $Ca^{2+}$, pH 4.0; *trace 5* □ 50 mM glucose + 0.1 mM $Ca^{2+}$ + 0.5 mM $Al^{3+}$, pH 4.0, ■ 50 mM glucose + 0.1 mM $Ca^{2+}$ + 5 mM $Al^{3+}$, pH 4.0, and ▲ 50 mM glucose + 0.5 mM $Al^{3+}$, pH 4.0. Times indicated are the 2-h periods during which each spectrum was obtained by time-averaging

with full aeration while sugar-phosphate levels remained relatively constant (within 10% of the starting level) up to 34 h of perfusion. Under hypoxic conditions, i.e. $N_2$ gas bubbling through the perfusate at pH 4.0, a significant change occurred in the cytoplasmic pH, to 6.5 as compared to 6.9 with a perfusate pH of 6.0 (Pfeffer et al. 1986). In addition, only a small increase of about 25% in cytoplasmic Pi was noted in contrast to the more than twofold increase observed when these experiments were conducted with an external pH above 6.0 (Roberts et al. 1984; Pfeffer et al. 1986).

*Effect of external pH on Pi generation.* A large amount of excess mobile Pi was generated in the root tips in the presence of glucose and 0.1 mM $Ca^{2+}$, at pH 4.0 as shown in Fig. 2A trace 1. An increase of approx. 100% of the initial level is observed after 34 h. The growth of the mobile Pi appears to proceed as a two-phase kinetics, rapid at first then followed by a slower process. A substitution of sucrose for glucose does not change the kinetic pattern of the growth as shown by the same trace. It should be mentioned that, as we reported previously (Pfeffer et al. 1986), there was a 35% increase in the signal for total mobile phosphorus and a 72% increase in Pi after 20 h when glucose was excluded and the perfusate contained only 0.1 mM $Ca^{2+}$ at pH 4.0.

Figure 2A trace 2 shows that an increase of the external pH to 7.0 alters the generation pattern so that only the slow near-linear kinetic process is operative. Thus, an increase of approx. 50% was obtained after 34 h. In all of the studies done at pH 4.0, the losses in nucleotide levels never exceeded 22% after 34 h, while at pH 7.0 the losses only reached a maximum of 12%.

*Effect of $Ca^{2+}$ on Pi generation.* The results illustrated in Fig. 2A indicate that the excess Pi production is favored by an acidic external environment and does not appear to be affected by replacing glucose with sucrose. However, the possible role of $Ca^{2+}$ in this process remains unanswered. In order to assess the kinetic role of $Ca^{2+}$, the experiments shown in Fig. 2B were performed. When $Ca^{2+}$ was omitted from the pH 4.0 medium (with glucose), the growth of the excess mobile Pi followed the pattern shown by trace 3 of Fig. 2B. Under the experimental conditions, an ever increasing rate of Pi appearance was observed. This pattern is consistent with an autocatalytic process. When the concentration of 5.0 mM $Ca^{2+}$ was used at pH 4.0, as shown in trace 4, the onset of the two-phase kinetics was delayed by an apparent induction period and final increase in Pi signal by 65%. Alternatively, the kinetic pattern may indicate a positive cooperativity of the process. These results indicate that $Ca^{2+}$ may play a minor regulatory role in the generation of the excess mobile Pi. A possible mechanism to account for the $Ca^{2+}$ regulation will be discussed.

*Effects of $Al^{3+}/Ca^{2+}$ on Pi generation.* In our previous study (Pfeffer et al. 1986), we reported that,

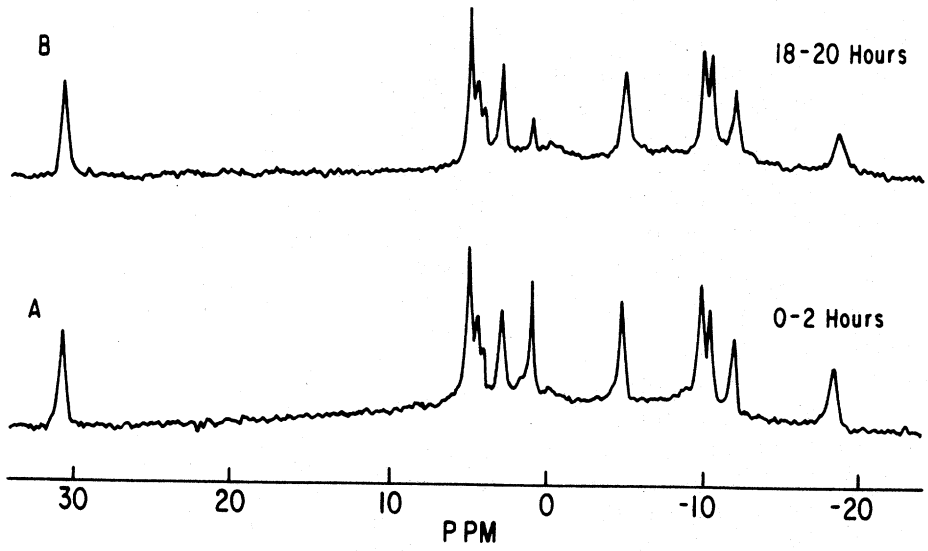


**Fig. 3A, B.** 161.7 MHz $^{31}P$ spectra of 3-d-old, 3–5 mm corn root tips perfused with a solution containing 50 mM glucose, 0.1 mM $CaSO_4$ and 2.5 mM $Al_2(SO_4)_3$ pH 4.0 for 0–2 h (**A**) and 18–20 h (**B**). Each spectrum was obtained as described in the legend of Fig. 1

in the absence of glucose and the addition of 0.1 mM $Al^{3+}$ and 0.1 mM $Ca^{2+}$ at pH 4.0, no additional Pi was produced. However, there was a significant decrease of nucleoside triphosphates (65%). This and the decreased respiratory activity, also previously reported, indicate a possible toxicity associated with the uptake of $Al^{3+}$. However, when glucose is supplied, the toxic effects of 0.50 mM $Al^{3+}$ on nucleotides and respiration are not observed (Pfeffer et al. 1986). What is observed, instead, is a significant depletion (40%) of soluble Pi as shown in Fig. 2B, trace 5. This is a much larger loss than is observed when only 5.0 mM $Ca^{2+}$ and glucose are present (Fig. 2B, trace 4). A combination of 0.1 mM $Ca^{2+}$ with 0.5 mM or 5 mM $Al^{3+}$ (1.0 mM $Al_2(SO_4)_3$ or 2.5 mM $Al_2(SO_4)_3$) in the presence of glucose gives a comparable result to the experiment carried out with 0.5 mM $Al^{3+}$ and glucose alone (Fig. 2B, trace 5). The spectra in Fig. 3 illustrate this phenomenon. Increasing the concentration of $Ca^{2+}$ to 5 mM in the presence of 0.5 mM $Al^{3+}$ does not suppress the effect of aluminum, i.e. we still observe a loss of approx. 40% soluble Pi after 18–20 h of perfusion of the tips (data not shown).

*Effects of $Al^{3+}$ under the conditions of hypoxia.* As mentioned before, the absence of glucose in aluminum-containing solutions appears to produce an impairment of metabolic processes in the maize roots. The omission of glucose in the perfusion solution presumably leads to a lower cellular energy status. An alternative way to lower the energy status of the root system is to replace $O_2$ by $N_2$. In the presence of 50 mM glucose an 0.1 mM $Ca^{2+}$, maize roots can survive hypoxia for up to 20 h even though nucleotide and sugar-phosphate levels remain reduced upon the reintroduction of aerobic conditions. At pH 4.0, these losses are somewhat higher than at pH 7.0 (see above). Treatment of roots with 0.1 mM $Ca^{2+}$, 0.5 mM $Al^{3+}$ and 50 mM glucose at pH 4.0 under hypoxia for 20 h resulted in a great loss of mobile vacuolar Pi (−93%), a 40% loss of sugar phosphates, and a 40% loss of nucleotides, following reaeration for 6 h. In contrast, in the absence of $Al^{3+}$, for the same time periods, only a 24% loss of Pi was observed while nucleotide levels decreased by 29% and sugar phosphates by an average of 20%.

*Root-surface phosphatase activity in the presence of aluminum.* In freshly prepared solutions of 0.25 mM $Al_2(SO_4)_3$, 0.1 mM $CaSO_4$ and 50 mM glucose we measured the change in phosphatase activity of excised root tips after incubation at room temperature for 24 h. These solutions consistently caused a 40% loss of phosphatase activity (0.006 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) when compared with the aluminum-free solution (0.010 $\mu mol \cdot mg^{-1} \cdot min^{-1}$). Solutions containing 0.25 mM $Al_2SO_4$ that were aged for 72 h prior to use with the excised tips showed no decline of phosphatase activity after 24 h of incubation.

*Examination of microbial contamination.* With regard to steps taken to eliminate the contributions of microorganisms, we performed each experiment in a perfusion system that had been thoroughly precleaned with NaOCl solutions. Each perfusate was prepared from highly deionized water. At

pH 4.0 no nitrogen source was present in the perfusate for any substantial microbial growth to develop. At pH 7.0, in the Mes-buffered solutions, microbial growth was somewhat higher after 24 h ($1\,600\,000 \cdot ml^{-1}$). However, our released phosphate as observed in the $^{31}P$ spectra was significantly lower, indicating that microbial-derived phosphatase activity was not responsible for the observed released soluble phosphate.

Without aluminum in the perfusate, the production of NMR-visible (soluble) Pi was essentially the same when glucose was or was not present at pH 4.0. However, microbial counts/ml (yeast plated on malt-extract agar) of the perfusate solutions containing 50 mM glucose or no glucose at time 0, 24 and 36 h were, in the order given 950/0, 250000/48000 and 500000/160000. The presence of 0.25 mM $Al_2(SO_4)_3$ in 50 mM glucose solutions at pH 4.0 had little effect on the microbial growth over 24 h (77000/ml), when compared to the aluminum-free solutions. The results of these experiments indicate that phosphatase activity is originating primarily from the plant tissue, presumably the cell walls as suggested by Crasnier et al. (1985), and not from the relatively minor level of microbial contamination found in the perfusate.

Results of $^{31}P$-NMR experiments performed without $Al^{3+}$ in the presence of 50 mM glucose and 0.50 mM $CaSO_4$ at pH 4.0 under aseptic conditions (i.e. all kernels surface-sterilized, all glassware and metal instruments sterilized (autoclaved) and the perfusion system cleaned with NaOCl solutions and flushed) were comparable to those run under non-aseptic conditions, i.e. they showed a 40% increase in soluble Pi after 16 h of perfusion with a microbial yeast count of only $25000 \cdot ml^{-1}$ after 32 h of perfusion.

## Discussion

In an earlier report (Pfeffer et al. 1986) we described the appearance of a mobile-Pi signal in the $^{31}P$ spectra of maize roots and suggested that this additional signal area arose from the hydrolysis of relatively immobile phosphorus-containing compounds, e.g. NMR-invisible forms as found in membrane phospholipids, nucleic acids or cell-wall-bound constituents. Such forms of phosphate, because of their long rotational correlation times, are not detectible with the normal high-resolution, solution NMR methodology used for in-vivo experiments (Pfeffer 1987). Thus they must be considered as NMR-invisible pools (Broedlius and Vogel 1985; Karczmar 1985). Our current results show that the formation of soluble, mobile Pi is favored at an acidic pH of the perfusion medium. Since the intracellular chemistry (nucleotide levels, cytoplasmic pH, etc.) is not noticably affected at this pH, the site of Pi generation is probably located in close proximity to the exterior environment. Since there is a good correlation of external pH with the production of this mobile Pi, it is possible that the release of some of the bound Pi may require the activation of a cell-wall acid phosphatase (McCain and Davies 1984).

While the form of carbohydrate used (glucose or sucrose) has little effect on the accumulation of this mobile Pi, $Ca^{2+}$ (0.1 mM and above) can regulate its appearance inside the cells. This is probably a reflection of a change in phosphatase activity and-or the membrane-transport property because of phosphatase $Ca^{2+}$ binding or a slight shift in the microenvironmental pH within the cell walls where Pi liberation is activated. When aluminum was in the presence of $Ca^{2+}$ in the ratios of 5:1, 20:1 and 1:10 respectively the spectra showed a large loss (35–50%) of tissue-mobile Pi primarily from the vacuolar compartment (Fig. 3). The same observation was made for glucose solutions containing only 0.25 mM $Al_2(SO_4)_3$ as well. It appears that both $Ca^{2+}$ and $Al^{3+}$ exert similar effects on the generation of soluble phosphate in maize root tips; however, the effect of aluminum seems to be much stronger and overshadows that of calcium when both ions are present. Aluminum ions may also inhibit the influx but not the efflux of Pi. This interpretation is consistent with the observations of Helyer (1978) that $Al^{3+}$ interfered with Pi uptake in legumes and of McCormick and Borden (1972) who observed cell-phosphate depletion by $Al^{3+}$ in barley.

With a high level of metabolism assured by an adequate supply of glucose, the energy status and intracellular pH of the cells are well maintained in the presence of both $Al^{3+}$ and $Ca^{2+}$. Thus $Al^{3+}$ appears not, as it does in the absence of an available carbohydrate source, to interfere with cytoplasmic metabolism (Pfeffer et al. 1986). Horst et al. (1983) and Matsumoto and Hirosawa (1979) have shown that aluminum penetration and inhibition of cell division and elongation in root apical meristems can occur under optimum respiratory conditions. In our studies of glucose-supplied tissue, nucleotide and sugar-phosphate levels appear to remain high; however, aluminum inhibition of cell division and elongation could still restrict the growth of vacuolar compartments as reservoirs for hydrolysed phosphate. This would favor migration of phosphate to the cell-wall regions where it could be precipitated with invading aluminum (McCor-

mick and Borden 1972). In addition, as mentioned above, the aluminum interference with phosphatase activity can also contribute further to the overall loss of the phosphate signal (McCain and Davies 1984). This is borne out in our phosphatase assay of aluminum- and non-aluminum-treated root tips. These experiments clearly demonstrate that freshly prepared 0.25 mM $Al_2(SO_4)_3$ solutions can inhibit phosphatase activity in excised root tips by 40% within 24 h as compared to aluminum-free solutions. In the absence of aluminum, liberated phosphate from NMR-invisible pools will freely fill the newly formed and expanding vacuoles giving rise to the observed increased vacuolar Pi signal.

In order to clarify the possible role of microorganisms in these studies we examined the $^{31}$P-NMR spectra of the plant tissue under aseptic conditions. At low levels of microbial contamination, in the absence of aluminum and presence of calcium and glucose we still observed the same production of soluble Pi as was observed under non-aseptic conditions, where microbial growth was relatively high. In addition, in the presence of aluminum, although microbial growth is only modestly reduced relative to the aseptic conditions, we consistently observed diminished levels of soluble-Pi production. Evidently, microorganisms (largely yeast at pH 4.0) are not contributing extensively to this observed phosphatase activity nor is the effect of the aluminum on the inhibition of microbial populations responsible for the loss of soluble Pi with time.

It has recently been shown by Rebeille et al. (1985) that an inadequate carbohydrate supply can cause severe metabolic declines in cultured sycamore cells (*Acer pseudoplatanus* L). Cells, already stressed at this level, may be much less able to maintain their energetic status with the invasion of aluminum. Consequently, the shutdown of certain pathways such as glycolysis (which has already undergone a substantial decrease because of lack of substrate) may be responsible for the observed rapid decline in cell metabolism. A report of Folsom et al. (1986) concerning $^{31}$P-NMR studies of aluminum-treated green algae indicates that the glycolytic pathway is possibly one of the prime targets for the toxicity of this metal.

Wagatsuma (1983) demonstrated that maize root tips were less resistent to aluminum toxicity under hypoxia. These results agree with our observations since following hypoxia, in the presence of aluminum, lower levels of nucleotides and sugar phosphates were observed relative to control experiments in which aluminum was absent. It seems clear that aluminum invasion of the cytoplasm has interfered with some metabolic processes. In addition, the severe loss of soluble Pi (approx. 93%) may be associated with a more alkaline cell-wall environment. That is, in the hypoxic state, a restriction of proton pumping and proton migration across the plasma membrane to the cell walls could facilitate more efficient cell-wall trapping of aluminum. Also, aluminum may have a degradative effect on membrane integrity during this vulnerable state of hypoxia; thus we might also anticipate a loss of Pi through leakage into the perfusion medium.

We thank Dr. David Bailey and Ms. Bonnie Murray for their help in performing the microbial analysis of the perfusate solutions.

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